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Manual

C.diff-Type AS-1 Kit

Array Hybridisation Kit for DNA-based detection of resistance genes and pathogenicity markers of *Clostridium difficile* and assignment of unknown *C. difficile* isolates to known strains

Kit order number: 246100096

96 reactions (**ArrayStrip** format)

For Research Use Only. Not for Use in Diagnostic Procedures.

CONTENT

BACKGROUND 1
GENERAL INSTRUCTIONS FOR USE 2
Intended Use 2
Specifications 2
Technical Support 2
Safety Precautions 3
Material Safety Data Sheets (MSDS) 3
Shipping Precautions 3
REAGENTS AND DEVICES 4
Kit Components, Storage and Stability 4
Cell Lysis (optional order) 4
DNA Labelling and Amplification 4
Hybridisation and Detection 5
Instrumentation and Software 6
Components Required but not Provided 6
PROTOCOLS 8
Culturing and Harvesting Bacterial Cells 8
DNA Extraction 8
DNA Extraction by Spin Columns (e.g. Qiagen) 9
DNA Extraction by Automated Device 11
Linear Amplification and Internal Biotin Labelling 11
Hybridisation 13
General Remarks - Handling of Arrays 13
General Remarks - Handling of Liquids 14
General Remarks - The Substrate (Precipitating Dye) D1 14
General Remarks - Thermoshakers 15
Protocol for Quantifoil's BioShake iQ 16
Data Analysis 18
Starting the ArrayMate Reader 18
Worklist 18
Data Acquisition in the ArrayMate Reader 20
Results 22
Export of C.diff-Type AS-1 Kit Test Reports 25
TROUBLESHOOTING 26
Staining Control 26
Image Quality 27

DNA Quality and RNA Contamination Control 27
Physical Damage to the Array 28
Report Unavailable 28
Ambiguous Results 28
Error Messages in Result Sheets 29
ADDITIONAL INFORMATION 30
Warranty 30
Disclaimer 30
Quality Control 31
List of Components for Separate Order 31
Legal Manufacturer 31
Contact 31
LITERATURE 32
UPDATES AND SOFTWARE 33
APPENDIX 1 – FLOW CHART 34
APPENDIX 2 – IMAGES FOR TROUBLESHOOTING 35
APPENDIX 3 – PROBE TO TARGET TABLE 38
APPENDIX 4 – TYPING INFORMATION 41
Definitions and Explanations 41
List of Currently recognized Strains 42

BACKGROUND

Clostridium difficile is a component of the human colonic flora. If the physiological bacterial flora in the colon is damaged by administration of antibiotics, especially of clindamycin, fluoroquinolones, cephalosporins, or amoxicillin/clavulanic acid, *C. difficile* is able to multiply and to cause antibiotic-associated diarrhoea and pseudomembranous colitis [1]. Severe cases might progress to toxic megacolon and end fatally. *C. difficile* can cause antibiotic-associated diarrhoea and a possibility of outbreaks in hospital settings warrants molecular typing.

The microarray based assay facilitates rapid and high-throughput genotyping of clinical *C. difficile* isolates including toxin gene detection and strain assignment. The **C.diff-Type AS-1 Kit** allows DNA-based detection of resistance genes and pathogenicity markers of *C. difficile* and assignment of unknown *C. difficile* isolates to known strains.

RNA-free, unfragmented genomic DNA from pure and monoclonal *C. difficile* colony material is amplified and internally labelled with biotin-dUTP using a linear amplification protocol. In contrast to standard PCR, only one antisense primer per target is used resulting in single stranded (ss) DNA reaction products. This allows a simultaneous sequence specific labelling and amplification of an essentially unlimited number of targets. However, sensitivity is lower than in a standard PCR (whereas contamination with undesired amplicons is nearly impossible) and for that reason the method is restricted to colony material and cannot be performed on samples such as swabs or pus. The resulting biotin-labelled ssDNA is transferred and hybridised to DNA oligonucleotide microarrays with 336 probes for different genetic markers and a biotin staining control. Most of them are printed in two duplicate spots.

Spot recognition is performed automatically based on digital images of the arrays. The overall pattern is analysed automatically for the presence or absence of specific genes and it is compared to a database of strain profiles allowing assignment to clonal complexes and strains.

GENERAL INSTRUCTIONS FOR USE

Intended Use

For Research Use Only. Not Intended for Use in Clinical Diagnostics.

This kit allows genotypic characterisation of bacterial cultures from *C. difficile* isolates for research and epidemiological applications. It must not be used as a substitute for phenotypic susceptibility tests and for the guidance of antibiotic therapy. It cannot be used for other bacteria than *C. difficile*.

Specifications

Upon receipt, the kit components need to be stored at different temperatures as specified on the package insert. The assay is to be performed at an ambient temperature of 18 °C to 28 °C.

Technical Support

If you require any further information on this product please contact:

Email: cct.home@clondiag.com

Phone: +49 (0) 36 41 3111-155

Fax: + 49 (0) 36 41 3111-120

For up-to-date information regarding the kit, please visit our website at

http://www.alere-technologies.com

Safety Precautions

The kit is intended for use by personnel that are trained in microbiological and molecular methods. Preparation of DNA from pure C. difficile colonies (clones) requires expertise in microbiology, and the local regulations for handling of pathogenic microorganisms (biosafety level 2) are to be obeyed.

Isolated, cell-free C. difficile DNA may be processed without further biosafety precautions, although contamination with C. difficile or other bacteria needs to be ruled out.

Always wear protective clothes as required for laboratory work by your local regulations.

Material Safety Data Sheets (MSDS)

According to OSHA 29CFR1910.1200, Commonwealth of Australia [NOHSC: 1005, 1008(1999)] and the latest regulations (EC) 1272/2008 (CLP) and 1907/2006 (REACH), the enclosed reagents do not require a Material Safety Data Sheet (MSDS), **except Hybridisation Buffer C1. The MSDS can be downloaded via our website from any lab solutions product page (e.g. http://alere-technologies.com/en/products/lab-solutions.html).** All other reagents do not contain more than 1 % of a component classified as hazardous and do not contain more than 0.1 % of a component classified as carcinogenic. Nevertheless, the buffers may cause irritation if they come into contact with eyes or skin, and may cause harm if swallowed. The regular precautions associated with laboratory work should be obeyed (e.g., wear protective goggles, gloves and lab coat and avoid contact with the reagents). If liquid is spilled, clean with a disinfectant and/or laboratory detergent and water.

Alere assumes no liability for damage resulting from handling or contact with these products. If you have any questions please contact our Technical Support (see above).

Shipping Precautions

RID/ADR: *Kein Gefahrgut* / No dangerous goods

IMDG: No dangerous goods

REAGENTS AND DEVICES

Kit Components, Storage and Stability

All reagents are provided in surplus (see below). If necessary, all components may be ordered separately. Please refer to the catalogue reference numbers (Cat#) at the end of this manual. For pricing please contact your local representative or our customer service, respectively.

The expiry date can be found on each bottle and on the outer packaging. All components were tested for short term shipment (< 1 week) at ambient temperature (< 37 °C). The assay components with limited stability are D1 and C3. The other kit components proved to be stable six months after post expiry.

Cell Lysis

- A1: Lysis Buffer
  Store at 18 to 28 °C (ambient temperature). Surplus: 50 %.
- A2: Lysis Enhancer (dried)
  Store at 18 to 28 °C (ambient temperature). Centrifuge A2 tubes shortly prior to opening. Add 200 µl Buffer A1 to Lysis Enhancer before use. Mix well and store for less than 1 week at 2-8 °C. Sufficient for 96 isolations.

DNA Labelling and Amplification

- $B1^{+}$: Labelling Buffer/Master Mix
  Store at 2-8 °C. Surplus: 25 %.
- B2: Labelling Enzyme
  Store at 2-8 °C. Surplus: 50 %.
- $B3^{Cdi}$: Primermix: dried Labelling Primermix, two tubes,
  dilute each primer mix in 70 µl molecular grade water. Store at -20 °C. Surplus: 50%

Hybridisation and Detection

- ArrayStrips (12 x 8 samples)

  Protected against light and sealed under inert gas. Store at 18-28 °C. After opening to be used within two weeks. Close the unused wells with caps, protect them against humidity and dust, and store them in a dark place. *Avoid any touching or scratching of the microarray surface at the bottom of the well. Do not store or handle unused wells at more than 60 % relative humidity since this may irreversibly corrode the spots.*

- StripCaps (24 units)

- **C1:** Hybridisation Buffer

  Store at 18-28 °C, protect against direct sunlight. Surplus: 100 %.

- **C2:** Washing Buffer 1

  Store at 18-28 °C, protect against direct sunlight. Surplus: 200 %.

- **C3:** HRP Conjugate 100 x

  Store at 2-8 °C, protect against direct sunlight. Surplus: 100 %.

- **C4:** Conjugate Buffer

  Store at 18-28 °C, protect against direct sunlight. Surplus: 200 %.

- **C5:** Washing Buffer 2

  Store at 18-28 °C, protect against direct sunlight. Surplus: 200 %.

- **D1:** Horseradish Peroxidase Substrate

  Store at 2-8 °C, protect against direct sunlight. Surplus: 50 %.

Instrumentation and Software

- ArrayMate Reader (to be ordered separately, for details see below)

  The ArrayStrip based **C.diff-Type AS-1 Kit** can be used on the ArrayMate reader only. The alternative devices ATR01/03 are not suitable for reading ArrayStrip based assays. In case of any questions please contact us.

- Iconoclust software (provided with the reader)

- Test specific software plug-in (can be downloaded from Alere Technologies GmbH website, check periodically for updates, for details see below). Information (such as spot names, marker names, location of the spots on the array, size of the image taken by the reader's specific camera) is delivered with the reader or can be downloaded from our website. These test specific plug-ins will occasionally be updated. Please check the NEWS section of our website http://alere-technologies.com/. Support is available via cct.home@clondiag.com.

Components Required but not Provided

- Growth media for the cultivation of *C. difficile* (for discussion of suitable media, see below).
- Equipment and consumables needed for the cultivation of *C. difficile* (incubator, anaerobic jars and catalysts, inoculation loops, Petri dishes)
- Additional assays for confirmation of species identification
- DNA preparation kit: The assay was tested with the DNeasy Blood & Tissue Kit from Qiagen (cat# 69504), QIAamp Minikit (cat# 51306) and a DNA preparation kit for Qiagen´s EZ1 automated device (DNA Tissue Kit, cat# 953034).

  Please note: DNA isolation from C. difficile requires a pre-treatment with the Cell Lysis components A1/A2 (see below).

- Equipment needed for DNA isolation, e.g., pipettes, centrifuge, thermoshaker or automated device (see above)
- Photometer for measuring the concentration of DNA

- Equipment for DNA gel electrophoresis for quality control of DNA
- Thermocycler
- Thermoshaker

  We strongly recommend the BioShake iQ by Quantifoil Instruments (http://www.qinstruments.com/) equipped with a customised heating block designed to fit ArrayStrips (# 312-010 | BioShake iQ - Alere® ArrayStrip). Alternatively, you may use Eppendorf's Thermomixer Comfort, equipped with a heating block for microtitre plates.
- Pipettes: suitable for volumes of 1 µL-5 µL, 90 µL, 100 µL, 200 µL, 1000 µL
- Multichannel Pipettes for 100-200 µL
- Reagent tubes suitable for PCR (VWR Cat# 732-0098)
- Ultrapure (PCR-grade) water
- Pasteur pipettes (VWR Cat# 612-2856)

PROTOCOLS

Culturing and Harvesting Bacterial Cells

C. difficile is a potential pathogen. All procedures for cultivation of the bacterium and DNA preparation need to be performed by properly trained staff in a biosafety level 2 facility.

C. difficile can be isolated under anaerobic growth conditions from faecal samples using pre-treatment with ethanol in order to kill off other bacteria and/or cycloserinethanol-cephoxitin-fructose-agar (e.g., Oxoid, Cat. Nr. PB5054A). Pre-reduction of the agar under anaerobic conditions or in an incubator with increased $C0_2$ concentration will improve recovery rates.

Since a comparatively high amount of DNA is required (because of the linear amplification, see below), single colonies from a primary culture usually do not suffice. Therefore, a single colony should be picked and used to inoculate several plates of blood or Schaedlar agar, or, more conveniently, a vial of Schaedler broth. The subculture should be incubated again (48 hrs at 37 °C and under anaerobic conditions), and cultures be harvested or, respectively, centrifuged.

- Centrifuge A2 tube briefly, open it, add 0.2 mL of Lysis Buffer A1 to Lysis Enhancer A2 and dissolve.
- Add some inoculatings loops full of monoclonal culture material of the *C. difficile* isolate, or the sediment of centrifuged liquid culture, into this A1/A2 reagent, vortex.

DNA Extraction

The required sample type for the **C.diff-Type AS-1 Kit** is 0.5-2 µg (c_{DNA}=0.1-0.4 µg/µl) of intact genomic DNA from a **single clone**.

This is much more DNA than for standard PCR applications (see Introduction).

The DNA specimen needs to be free of RNA and it should not be fragmented.

This can be determined by agarose gel electrophoresis. DNA should not be prepared by disrupting *C. difficile* cells using bead beaters, ultrasonication or aggressive chemicals such as in alkaline lysis protocols. Most performance problems with the **C.diff-Type AS-1 Kit** are due to insufficient amounts or quality of DNA preparation. We therefore strongly recommend obeying the protocols outlined below.

DNA Extraction by Spin Columns (e.g. Qiagen)

- Centrifuge A2 tube shortly, open it, add 0.2 ml of Lysis Buffer A1 to Lysis Enhancer A2 and dissolve.
- Add culture material of the *C.difficile* isolate (as described above) to this A1 / A2 reagent and vortex thoroughly.
- Incubate the culture material of the *C.difficile* isolate in A1 / A2 for 30-60 min at 37 °C and 550 rpm in the thermoshaker.
- Proceed with the DNA preparation protocol of the DNA preparation kit. For the Qiagen DNeasy Blood&Tissue Kit it is as follows:
- Add 25 µl proteinase K (from Qiagen Kit, or equivalent) and add 200 µl buffer AL (Qiagen Kit).
- Vortex shortly or shake vigorously.
- Incubate for 30-60 min at 56 °C and 550 rpm in the thermoshaker.
- **Important**: If A1/A2 reagent is not used, add now 4 µl RNase A (100 mg/ml), mix by vortexing, and incubate for 2 min at room temperature before continuing.
- Add 200 µl ethanol (96 - 100 %).
- Vortex the sample and centrifuge (quick spin).
- Transfer the complete tube content (including any precipitate) into a spin column that is placed in a 2 ml collection tube.
- Centrifuge (8,000 rpm, 1 min) at room temperature. Time and speed need to be determined depending on the sample viscosity and the type of centrifuge used. All liquid should be collected in the collection tube afterwards.

- Discard collection tube with liquid.
- Place the spin column in a new 2 ml collection tube (provided with the kit).
- Add 500 µl Buffer AW1.
- Centrifuge (8,000 rpm, 1 min) at room temperature.
- Discard collection tube with liquid.
- Place the spin column in a new 2 ml collection tube (provided with the kit).
- Add 500 µl Buffer AW2.
- Centrifuge (14,000 rpm, 3 min) at room temperature. The membrane of the spin column should be dry, and all liquid should be in the collection tube.
- Discard collection tube with liquids.
- Place the spin column in a clean 1.5 ml tube (not provided with the kit).
- Add 100 µl Buffer AE (or PCR grade distilled water) directly onto the membrane of the spin column.
- Incubate at room temperature for 1 min to elute DNA.
- Centrifuge (8000 rpm, 1 min) at room temperature.
- Optional: Add another 100 µl Buffer AE (or PCR grade distilled water) directly onto the membrane, incubate at room temperature for 1 min and centrifuge again.
- Discard the spin column.

Please note: Ethanol from Washing Buffers strongly inhibits the enzymes used in the assay. Contamination with Washing Buffer might occur during the elution of prepared DNA by drops adhering to the spin columns funnels. Therefore these funnels should be gently touched and dried with sterile filter paper or wipes prior to the elution step. Alternatively, prepared DNA can be heated shortly to evaporate ethanol (e.g. 10 min at 70 °C).

- Check for DNA integrity and absence of RNA (e.g. agarose gel). If necessary, you might perform another digestion step with additional RNase A (not provided). Measure DNA concentration (A_{260} method); it should not be lower than 0.1 µg / µl. The concentration might be increased by heating and evaporating water, or by using a speed vac centrifuge (not recommended when the same preparation shall be used in PCR experiments).

DNA Extraction by Automated Device

The assay was tested with Qiagen´s EZ1. Other systems also can be used as well. However, performance should be checked with some known reference strains prior to routine use. Incubate the colony material of the *C. difficile* isolate in A1/A2 for 30-60 min at 37 °C and 550 rpm in the thermoshaker as described above (depending on the input sample volume required by the device you actually use, the A1/A2 mixture might be divided into two aliquots, and used for DNA preparation of two samples).

- Add 10 µL proteinase K and add 100 µL buffer AL.
- Vortex briefly or shake vigorously.
- Incubate sample 45-60 min at 56 °C and 550 rpm in the thermomixer.
- When the cells are lysed, proceed by performing the tissue lysis protocol (Bacteriacard) for Qiagen´s EZ1
- *For Qiagen´s EZ1*: Front row: empty elution tubes (1.5 mL); second row: tip holder with tips; third row: empty; back row: sample tube with conical tip (2 mL) containing the 200 µL sample volume. Set tissue lysis protocol with a set sample volume of 200 µL and an elution volume of 50 µL.
    - Concentrate DNA and evaporate traces of solvents by heating the sample at 70 °C for 5-10 min.

Linear Amplification and Internal Biotin Labelling

Please keep in mind the limited surplus of reagents whilst pipetting. The surplus of $B1^+$ labelling reagent is 40%.

- Prepare a Master Mix by combining 3.9 µL of $B1^{+}$ labelling reagent, 0.1 µL of B2 labelling enzyme and 1.0 µl of $B3^{Cdi}$ primer mix per sample.
- Add 5 µL DNA (0.5-2 µg) prepared as described above to a 5 µL aliquot of the Master Mix. Do not forget to label the vial!
- Perform amplification in a pre-programmed thermocycler (such as Mastercycler gradient with heated lid, VWR, cat# 460-0108) according to the following protocol:

Pre-heat cover / lid to 105 °C	
300 sec at 96 °C	
55 cycles with:	60 sec at 96 °C
	20 sec at 50 °C
	40 sec at 72 °C
Cool down to 4 °C, hold	

- The amplification products can be stored frozen until usage.

Please note: When using a different device, some adaptations, such as an increase of the number of cycles, might be necessary. Before establishing routine use, please test the protocol with a few known reference strains or the control DNA (CM) supplied upon request. We recommend as reference strain the following:

Clostridium difficile

DSM No.: 27543

Strain designation: 630

https://www.dsmz.de/catalogues/details/culture/DSM-27543.html

Hybridisation

General Remarks - Handling of Arrays

- *Never touch the array surface!*
- *Avoid complete drying of the array surface during processing!*
- *Do not allow it to stay without liquid for more than two minutes!*
- *Never rinse the wells with distilled water after the hybridisation step, only use C2 Washing Buffer!*

Unused wells should be capped during the whole procedure. The strips may be processed up to three times without a loss of quality of properly capped unused arrays. Close all wells that will not be used with a cap und leave them there until you use these wells (for storage conditions after use: see section “Kit components, Storage and Stability / Hybridisation and Detection”).

Always label your ArrayStrips with a laboratory marker at the recommended position. **Never** label them on the bottom or across the data matrix barcode! This may cause errors.

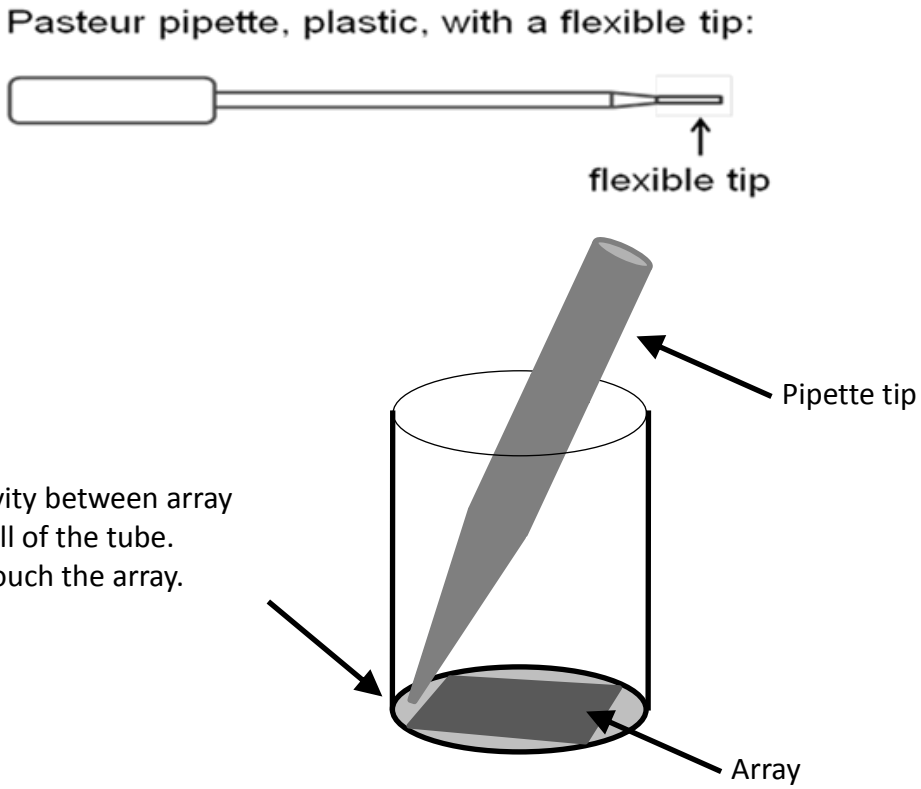
Avoid contact of data matrix barcode with organic solvents! The ArrayMate needs the information encoded in the data matrix to perform the assay and the analysis afterwards.

Avoid touching the bottom of the microarray strip and keep it clean.

General Remarks - Handling of Liquids

We recommend the use of a multichannel pipette and reagent reservoirs.

We strongly recommend that the liquid is removed by pipetting rather than by inverting the strips and flicking the liquids out. Fine tipped soft, disposable Pasteur pipettes are suited best (such as VWR / Cat# 612-2856). Always place the pipette tip at the cavity between the array and the wall of the reagent well. If you touch the array surface, probes may be scratched off and this may cause errors.


Pasteur pipette, plastic, with a flexible tip:
flexible tip
Pipette tip
Use the cavity between array
and the wall of the tube.
Do never touch the array.
Array

General Remarks - The Substrate (Precipitating Dye) D1

An appropriate amount of D1 substrate (precipitating dye) should be transferred into an Eppendorf tube and taken out of the refrigerator when starting the procedure allowing it to acclimatise to room temperature (25 °C). Cold D1 may yield weak signals. D1 should be centrifuged prior to use to remove bubbles as well as possible precipitates (quick spin).

Triggered by peroxidase, the dye precipitates in case of positive reactions, but it is not covalently bound. The precipitate can be dissolved by vigorous shaking. Thus, the arrays must **not be shaken**, dropped or moved abruptly during the staining procedure or thereafter.

After completion of staining, remove and discard reagent D1 as completely as possible and scan immediately (ArrayMate). The dye precipitate fades slowly in presence of liquids.

General Remarks - Thermoshakers

The correct temperature within the vessels is essential; therefore always use the appropriate equipment for heating. Because of a possibly inhomogeneous distribution of the temperature within the heating block and because of possible differences between displayed and actual temperatures, the use of different brands of thermoshakers might affect test performance. We tested the assay with BioShake iQ by QInstruments (see picture below) (http://www.qinstruments.com/) equipped with a customised heating block designed to fit ArrayStrips and Eppendorf's Thermomixer Comfort, equipped with a heating block for microtiter plates. When using other devices, some modifications to the protocol might be necessary. Before starting routine use, please test the protocol with a few known reference strains or the control DNA (CM). The difference between the protocols for QInstrument´s BioShake iQ and Eppendorf's Thermomixer Comfort with microtiter plate adapter is only the washing temperature after the hybridisation step.

Please note: The Quantifoil's BioShake iQ has no active cooling function. Please use the second pre-temperatured passive cooling-block to reduce the incubation temperature quickly.

Protocol for Quantifoil's BioShake iQ

BioShake iQ by QInstruments equipped with a customised heating block designed to fit ArrayStrips.
http://www.qinstruments.com/

- Switch on the thermoshaker and let it pre-heat to 50 °C.
- Remove the amount of ArrayStrip(s) needed from the pouch.
- Insert the ArrayStrip(s) into the white frame. Assure the correct orientation (data matrix barcode close to row A) and proper fit.
- Pre-wash the array(s) in two steps:
  - First, PCR-grade distilled water, 200 µl per well at 50 °C, 5 min at 550 rpm. Remove the water from the well.
  - Second, C1 Hybridisation Buffer, 200 µl per well at 50 °C, 5 min at 550 rpm.
- Add 90 µl of C1 buffer to each tube with 10 µl labelled amplification product, mix gently.
- Remove the buffer from the well and add the mixture of C1 and labelled amplification product.
- Incubate at 50 °C, 60 min at 550 rpm.
- **Meanwhile, login to the ArrayMate device and prepare your worklist (see section "Data Analysis" p. 20)**
- Remove the liquid and add 200 µl C2 Washing Buffer. Incubate at 45 °C, 10 min at 550 rpm, remove and discard.

- Add another 200 µl C2 Washing Buffer. Incubate at 45 °C, 10 min at 550 rpm.
- Meanwhile, prepare conjugate: For each experiment add 1 µl C3 conjugate 100 x HRP to 99 µl C4 Conjugation Buffer. This mixture is stable for one working day at room temperature; C3 is delivered with a surplus of 100 %, C4 with a surplus of 200 %.

  Suggested pipetting scheme:

	1 well	2-3 wells	4-6 wells	7-10 wells	11-15 wells	16-20 wells	21-30 wells	31-40 wells
C3	1.5 µL	3.5 µL	7 µL	11 µL	16 µL	21 µL	32 µL	42 µL
C4	148.5 µL	346.5 µL	693 µL	1089 µL	1584 µL	2079 µL	3068 µL	4058 µL

- Remove the Washing Buffer, and add 100 µl diluted conjugate to each well, incubate at 30 °C, 10 min at 550 rpm.
- Remove the conjugate (C3 / C4), add 200 µl C5 Washing Buffer. Incubate at 30 °C, 5 min at 550 rpm.
- Remove the Washing Buffer, add 100 µl of D1 (HRP substrate, precipitating dye, at 25 °C, see above) per well.
- Incubate at 25 °C for 10 min *but* ***do not shake!***
- Remove liquid completely.
- The bottom of the ArrayStrips (outside surface) may be cleaned cautiously with wipes. Bubbles may be removed by removing and adding D1.
- Scan and process (ArrayMate, see below).

Please note: *Check immediately all images for cleanliness (i.e., absence of dust particles, residual liquids) and for good focus. Dust particles and residual fluids inside the vial can be removed by cautiously washing twice with 200 µl PCR-grade distilled water. If necessary, scan and process again (For Troubleshooting see p. 26 and 35-37).*

Data Analysis

Starting the ArrayMate Reader

We recommend starting the ArrayMate Reader after starting the hybridisation; this allows the convenience of starting the device and to importing the worklist file.

Please note that this is a short instruction only. For more detailed information please refer to the ArrayMate User Manual.

- Switch on the ArrayMate (1st: main switch on the rear below the electric cable plug, 2nd: operating switch on the bottom left corner of the front side).
- Switch on the screen (switch is on the right hand side below the screen).
- Log-in as **R&D User** (Research and Development User) for full access to test specific software (a default password will be provided together with the ArrayMate device). If you log-in as **User**, you will obtain only raw values, but neither positives/negatives interpretation nor strain assignment. The **Administrator** log-in will allow the installation of a new assay specific plug-in, which can be downloaded at http://alere-technologies.com (see p. 31).
- The user interface will be loaded, the ArrayMate performs internal testing. It requires slightly less than a minute.
- Click **New Run** (left upper edge of the screen). A suggestion for a run name/folder name for the new run appears in the top line of the screen. You may modify or change the experiment name at your convenience.
- Type in your operator ID (**optional**).

Worklist

A **Worklist** file allows linking an identifier such as a laboratory or sample number to a position of an array within the ArrayStrip. For privacy reasons, arrays should not be identified by patient names. Worklists can be generated using spreadsheet software such as EXCEL (see below) but

must be saved in the *.txt file format that can be imported into the test-specific ArrayMate software. **Do not use special characters** (such as: ; ()[] / \ ä ü etc.).

- Create a list with at least three columns that have headers written in the first line. The following headers are obligatory (in this order): position / sampleID / assayID (Table 1).
- Positions are consecutively numbered from 1 to a maximum of 96. Position 1 would correspond to A1, 8 to H1, 9 to A2 and 96 to H12 (Table 2). Do not leave empty lines in the worklist. If you use EXCEL, position numbers should be entered into column A.
- Sample IDs are strain/sample/laboratory numbers such as exported from your LIMS (or assigned in any different way). Patients' names should not be used as sample IDs.
- The Assay ID allows the system to identify the current test and to correctly use information on layout, spot number, and identity etc. The **E C.diff-Type AS-1 Kit** has the Assay ID: **16045**. ***Please note:*** *Assay ID numbers must not be confused as this could lead to errors or loss of data.*
- You may add further columns and headers with notes and comments at your convenience. Information from these columns will not appear on the result screen or in the Test Report.
- We recommend using a printout of the worklist as a template for pipetting.
- Save the worklist as **tab separated *.txt** file on the memory stick provided together with the ArrayMate.
- To avoid confusion, make sure that worklists are named unambiguously or that worklists from earlier experiments are deleted.
- You may use the software tool **Worklist Generator** to create a worklist easily.

  http://alere-technologies.com/en/products/lab-solutions/software-tools/worklist-generator.html

Table 1: Example worklist. *Please note: Table header must be written exactly as shown.*

position	sampleID	assayID
1	2015-12345	16045
2	2015-12346	16045
3	2015-12347	16045
4	2015-12348	16045
5	2015-12349	16045
6	2015-12350	16045
7	987654	16045
8	*C.diff.*	16045

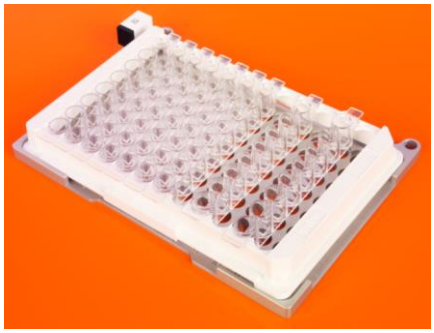
Table 2: Positions in the 96 well format

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
H	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Data Acquisition in the ArrayMate Reader

- Insert your flash drive containing the worklist into any of the USB ports on the lower right-hand side of the ArrayMate.
- Press ; a folder selection dialogue will open.
- Select your worklist (path: ‘My Computer/Removable Disk’).
- Open your selected worklist by pressing **Enter** or **Open**.
- Press (your imported worklist opens in a separate window). Proofread. If the new window is empty, or if it was the wrong worklist, repeat the import.
- Press **OK**; the worklist window will close.

- Leave the flash drive in the ArrayMate if you intend to export **C.diff-Type AS-1 Kit** reports afterwards (Check the flash drive regularly for computer viruses and malware using an appropriate program.).
- Press **Next** (at the bottom right on the screen; reader is opening).
- Carefully insert the appropriate metallic adapter/frame into the ArrayMate. Do not apply strong force. Ensure proper fit, otherwise the images may be out of focus.
- Carefully insert the white frame with the array strips into the metallic adapter. Ensure the correct orientation (Position A1 in the frame next to the data matrix barcode on the adapter) and proper fit; otherwise the images may be out of focus.



ArrayStrip frame with inserted strips. Strips are inserted in accordance with the **Worklist**.

Please note: ArrayStrips must be clean. They should not contain any liquids during analysis. Data matrix codes must be clean. There must be no Array StripCaps on the wells to be analysed (however, unused wells should remain capped).

- Press **Next** (at the bottom right on the screen; reader closes, analysis program starts, it takes about 2-10 min, depending on the number of strips; the reader takes images and automatically analyses the data). The progress of the reading is indicated by the following symbols:

photographed: in analysis: ready:

- The reader indicates the end of the entire process with an acoustic signal (beep).
- Press **Next** (at the bottom right on the screen; reader is opening).
- Remove the white frame with the ArrayStrip(s).

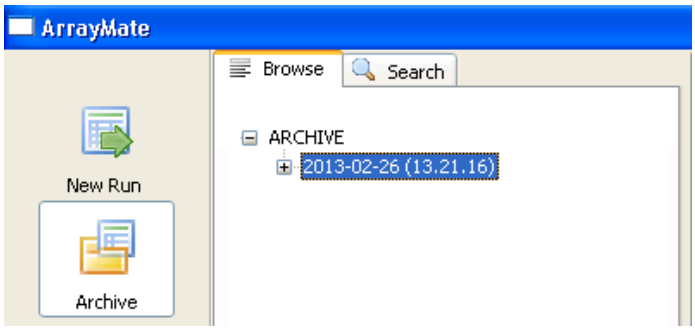
- Press **Next** (at the bottom right on the screen; reader is closing).

Results

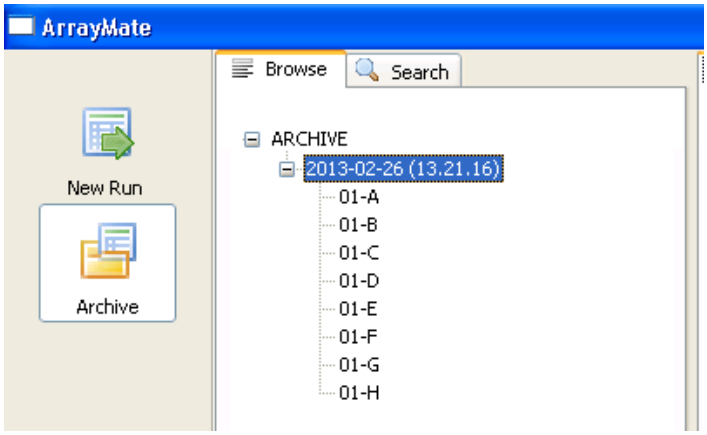
On the left-hand side of the screen, there you will see a list showing all runs stored on the ArrayMate´s hard disk. A run contains the results from all arrays analysed together within one frame. If this list is not displayed:

- Press **Archive** (left hand side) and activate the flag **Browse** (at the top left).
- The runs are organised like folders in **Windows Explorer,** and named **by default** according to the date of acquisition.

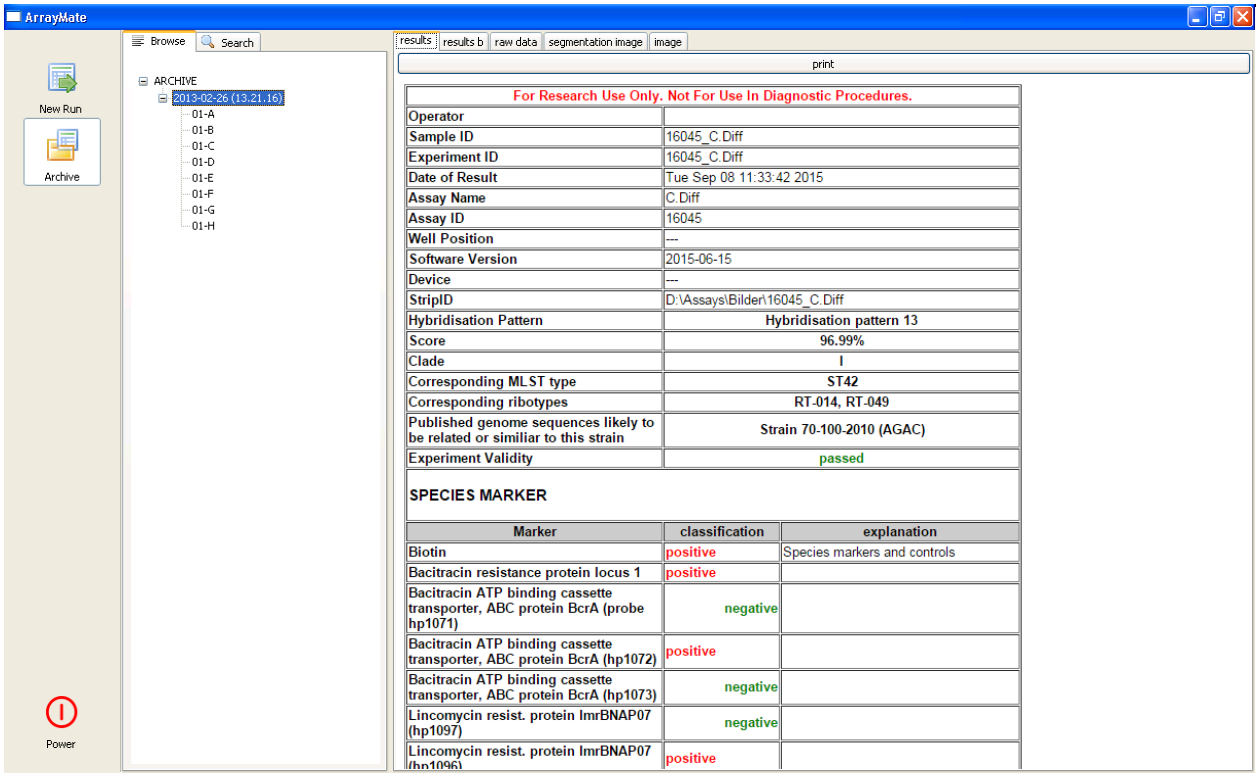
Example: There is one experiment run in this archive:



If you click on the plus symbol left on the run name, the folder opens and you will see a list of the individual arrays ordered by Sample ID.



Click on a Sample ID, and the **C.diff-Type AS-1 Kit** test report for this array is shown in the window on the right:



For Research Use Only. Not For Use In Diagnostic Procedures.	
Operator	
Sample ID	Jena_0016_11 - {E55CD746-D060-4C75-9706-8524558DC191}.16045_8.chip
Experiment ID	Jena_0016_11 - {E55CD746-D060-4C75-9706-8524558DC191}.16045_8.chip
Date of Result	Tue Jul 14 12:50:35 2015
Assay Name	C.diff
Assay ID	16045
Well Position	---
Software Version	2015-06-15
Device	---
StripID	T:\InesE\Jena_0016_11 - {E55CD746-D060-4C75-9706-8524558DC191}.16045_8.chip
Hybridisation Pattern	**Hybridisation pattern 35**
Score	**94.74%**
Clade	**IV**
Corresponding MLST type	**ST-37, ST-86**
Corresponding ribotypes	**RT-017**
Published genome sequences likely to be related	**CF5 (FN665652), 002-P50-2011 (AGAA), 050-P50-2011**

or similiar to this strain	**(AGAB), M68 (FN668375)**	
Experiment Validity	**passed**	
SPECIES MARKER		
Marker	**classification**	**explanation**
Biotin	**positive**	Species markers and controls
Bacitracin resistance protein locus 1	**positive**	
Bacitracin ATP binding cassette transporter, ABC protein BcrA (probe hp1071)	**positive**	
Bacitracin ATP binding cassette transporter, ABC protein BcrA (hp1072)	**negative**	
Bacitracin ATP binding cassette transporter, ABC protein BcrA (hp1073)	**negative**	
Lincomycin resist. protein lmrBNAP07 (hp1097)	**negative**	
Lincomycin resist. protein lmrBNAP07 (hp1096)	**positive**	
Channel-forming haemolysin	**positive**	
Efflux pump ydiC	**positive**	
Putative lantibiotic ABC transporter, permease protein (hp1251)	**positive**	
Putative lantibiotic ABC transporter, permease protein (hp1252)	**negative**	
TOXINS		
Marker	**classification**	**explanation**
Toxin A detection	**positive**	
Toxin A allele	**tcdA-CF5**	
Toxin B detection	**positive**	
Toxin B allele	**tcdB-CF5**	
ANTIBIOTIC RESISTANCE		
Marker	**classification**	**explanation**
cat	**negative**	chloramphenicol acetyltransferase
ermB	**negative**	rRNA methyltransferase
tetM	**negative**	tetracycline resistance protein
sat (hp1082)	**positive**	streptogramin A acetyltransferase
sat (hp1083)	**negative**	
OTHER VIRULENCE GENES		
Marker	**classification**	**explanation**
Binary Toxin (cdtA/B) detection	**negative**	Binary Toxin (Total)
Binary Toxin (cdtA/B) allele	**negative**	
tcdE (hp1113)	**positive**	holin-like pore-forming protein
tcdC (hp1117)	**ambiguous**	putative negative regulator of pathogenicity locus

Export of C.diff-Type AS-1 Kit Test Reports

Two result files in htmL format will be generated. The shorter report will gives a summary on clinically relevant genes (virulence markers and genes associated to antibiotic resistance) and typing information.

This includes the affiliation to strains with unique hybridisation patterns and to MLST-defined clades. MLST sequence types and ribotypes known to be associated with the respective hybridisation pattern are also displayed as well as related/identical genome sequence. Note that information on sequence types and ribotypes is derived from a database search, not from an actual experiment. We are grateful if you could share additional information on sequence types and ribotypes as this could help to improve the software in future.

A longer htmL result sheet ("result_B.res.htmL") provides information on all probes.

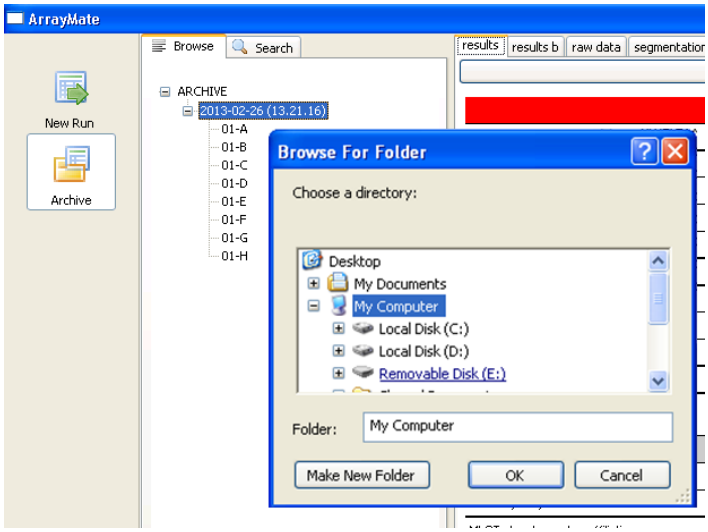
Possible error messages in these reports will be explained below (see Troubleshooting).

Other files that are generated and that can be exported include

- A *.txt file with the raw measurements,
- An image file (*.bmp) showing the actual picture of the array,
- A second image file (*.png) in which the coordinate grid is superimposed and the recognised spots are circled, and
- A *.xmL file providing the same information as the htmL result sheet for future export into databases and for using the **Result Collector** tool.
- An *.out file containing output log data which helps our service to trace image evaluation errors

Please Note: Only complete runs can be exported. The export of individual **C.diff-Type AS-1 Kit** *Test Reports is not possible.*

- Right-click on the selected run (a menu appears with the option **Export Run Reports**).
- Right-click on **Export Run Reports** (a file browser opens).



- Click on **My Computer**, subsequently on **Removable Disk,** and choose the folder where to save or click on the button **Make New Folder** (on the bottom; a new folder icon appears).
- Rename the new folder (e.g. with the experiment name or date).
- Click on the **OK** button (data are exported into the new folder on your memory stick).
- Do **NOT** remove the memory stick as long as the hourglass symbol is visible.
- Switch off the device by clicking on the **Power** button (left / down on the screen):

- Switch off the Screen. There is no need to physically switch off the ArrayMate Reader.

TROUBLESHOOTING

In case of trouble always make sure that reagents are within the recommended shelf-life and stored under appropriate conditions.

Should you encounter a problem, we will always be happy to support you. Please e-mail to cct.home@clondiag.com and include a description of the problem as well as the array images (*.bmp files) in question.

Staining Control

A staining control is included to check whether possible problems originate from the hybridisation or the staining procedure. If the staining control has “Failed” proceed as follows:

Horseradish peroxidase conjugate may have degraded during storage. Add 1 µl buffer C3/C4 to 9 µl D1 (substrate). If the solution turns green within 3-5 seconds, the horseradish peroxidase still has sufficient enzymatic activity.

Enzymatic reaction is inhibited by carryover of buffer C1. Ensure proper washing of the wells with C2 buffer to remove all C1 buffer prior to adding horseradish peroxidase conjugate.

If the staining control has "Passed", refer to the following hints.

Image Quality

In case of poor image quality we recommend to re-check DNA quantity and quality first by loading leftover DNA on an agarose gel.

In order to determine whether any problems originated from the DNA preparation, perform an experiment with the Control material (CM). This is DNA from the *C. diff.* reference strain (details, strain ID, corresponding GenBank number etc. upon request). If the control experiment yields a valid result and a correct identification, there was probably an issue with DNA preparation. If the control experiment also fails, an error affecting later steps or a degradation of reagents from later steps is likely.

See also Appendix 2 – Images for troubleshooting (p. 37 - 39).

DNA Quality and RNA Contamination Control

The amount of DNA is crucial because of the linear kinetics of amplification (see Introduction). DNA should be free of RNA, as RNA reduces the efficiency of amplification and labelling by effectively removing primer from the reaction mix due to competitive hybridisation. A_{260} readings will cover RNA and other contaminants as well. Therefore pure DNA preparations without RNA contamination are prerequisite for proper DNA concentration measurement. RNAse treatment prior to A_{260} reading therefore is necessary (component A2 contains RNase).

DNA must be unfragmented, as fragmentation reduces the efficiency of amplification and labelling due to the distance between primer and probe binding sites. For this reason DNA should not be prepared by disrupting *C. difficile* cells using bead beaters, ultrasonication or

aggressive chemicals such as in alkaline lysis protocols. We gained positive experiences with the manual QIAGEN DNeasy Kit and the automated device EZ1.

DNA must be free of any trace of ethanol, as ethanol strongly influences the amplification. It is possible to heat the sample prior to adding it to the Labelling Primermix (5-10 min at 70 °C). Some problems with samples from the Qiagen EZ1 device for example were resolved after heating the samples (see above).

Physical Damage to the Array

Scratching the array surface with a pipette tip may damage array spots, which may lead to the impairment or absence of a valid signal. In this case, the respective marker will not be assigned as 'negative', but instead, the message 'none' appears next to the marker name.

Report Unavailable

If the ArrayMate indicates that no report is available for an array (or multiple arrays on one strip), please check that the strip was positioned properly into the frame. Scratches or drops of condensed water might render the Data Matrix code identifier unreadable, please wipe it carefully or try to manually identify the test.

If no obvious reason for the fault can be discovered, please contact the technical service.

Ambiguous Results

Apart from a "positive" or "negative" result for the individual markers on **C.diff-Type AS-1** Test Report, the result can also be "ambiguous".

In cases affecting resistance genes or virulence factors, no definitive answer with regard to the presence of this specific marker than can be given. This can be caused by poor sample quality, poor signal quality and, in case of some resistance-associated genes by the presence of plasmids in low copy numbers.

Allelic variants of some markers differ only in single or few nucleotides. This can cause the effect that the actual allele yields a positive signal while other, mismatching probes give ambiguous rather than negative results.

Error Messages in Result Sheets

Please compare Appendix 2 for the possible error messages in Result Sheets and for corresponding array images.

ADDITIONAL INFORMATION

Warranty

Alere Technologies GmbH guarantees the performance as described in this manual. Usage of the kit was successfully tested at ambient temperatures up to 37 °C. A guarantee is limited to ambient temperatures in the laboratory between 18 °C - 28 °C. Kit components comprise the arrays and their caps, the Lysis Enhancer, the reagents for DNA labelling and for detection of labelled DNA products on the array, the ArrayMate reader and its software. In case one of these components fails within the expiry date due to other reason than misuse, contact Alere Technologies GmbH for replacement or refund. Terms and conditions apply.

If you have any problem or question, please contact the technical service.

Disclaimer

This system is for research use only.

We do not accept any liability for damages caused by misuse. Misuse comprises, especially but not exclusively, of a use of the system for the detection of resistance genes in order to predict phenotypic antibiotic resistances or susceptibilities for the guidance of an antibiotic chemotherapy.

Since resistances might be caused by genes or mutations not covered by this array or by hitherto unknown genes or mutations, any antibiotic chemotherapy MUST be guided by phenotypic susceptibility tests.

Furthermore, we do not accept any liability for damages caused by inappropriate use of the device as a personal computer, for instance related to the use of additional software, to network connections, or to a breach of privacy related to the storage of confidential information (such as names of patients from whom *C. difficile* was isolated) on its hard disk and/or to the use of external storage devices that might be contaminated with spyware.

Quality Control

Each batch is stringently tested for good performance and correctness of results using standard *C. difficile* DNA preparations.

List of Components for Separate Order

If required, these reagents for the **C.diff-Type AS-1 Kit** may be ordered separately:

component	name	amount	cat#	storage
A1	Lysis Buffer	30 ml	245101000	18-28 °C
A2	Lysis Enhancer	96 units	245102000	18-28 °C
$B1^{+}$	Labelling Buffer	550 µl	245201000	2-8 °C
B2	Labelling Enzyme	20 µl	245104000	2-8 °C
$B3^{Cdi}$	C.diff Primermix	70 µl / tube	246103500	2-8 °C
C1	Hybridisation Buffer	30 ml	245105000	18-28 °C
C2	Washing Buffer 1	120 ml	245106000	18-28 °C
C3	HRP Conjugate 100x	200 µl	245107000	2-8 °C
C4	Conjugate Buffer	30 ml	245108000	18-28 °C
C5	Washing Buffer 2	120 ml	245109000	18-28 °C
D1	HRP Substrate	15 ml	245110000	2-8 °C
CM^{Cdi}	*C. diff*. DNA (c_{DNA} = 0.1-0.4 µg/µl)	30 µl	246111000	2-8 °C
ArrayStrip	C.diff-Type AS-1	1 strip	240009610	15-28 °C
StripCap	StripCap	24 units	245112000	15-28 °C

For pricing please contact your local representative or our customer service, respectively.

Legal Manufacturer

Alere Technologies GmbH

Loebstedter Str. 103-105

07749 Jena, Germany

Contact

If you require any further information on this product please e-mail to cct.home@clondiag.com

LITERATURE

Lubbert C, John E, von Muller L (2014) Clostridium difficile infection. Dtsch Arztebl Int 111 (43):723-731

Dingle KE, Griffiths D, Didelot X, Evans J, Vaughan A, Kachrimanidou M, Stoesser N, Jolley KA, Golubchik T, Harding RM, Peto TE, Fawley W, Walker AS, Wilcox M, Crook DW (2011) Clinical Clostridium difficile: clonality and pathogenicity locus diversity. PLoS ONE 6 (5):e19993

Gawlik D, Slickers P, Engelmann I, Müller E, Lück C, Friedrichs A, Ehricht R, Monecke S. (2015) DNA-Microarray-based Genotyping of Clostridium difficile. BMC Microbiol. doi: 10.1186/s12866-015-0489-2. PMID: 26242247

http://www.biomedcentral.com/1471-2180/15/158

For further literature please refer to:
http://alere-technologies.com/en/science-technologies/publications/downloads.html.

UPDATES AND SOFTWARE

Notifications on database/software updates and freeware tools can be found at:

http://alere-technologies.com/en/products/lab-solutions/clostridium-difficile.html

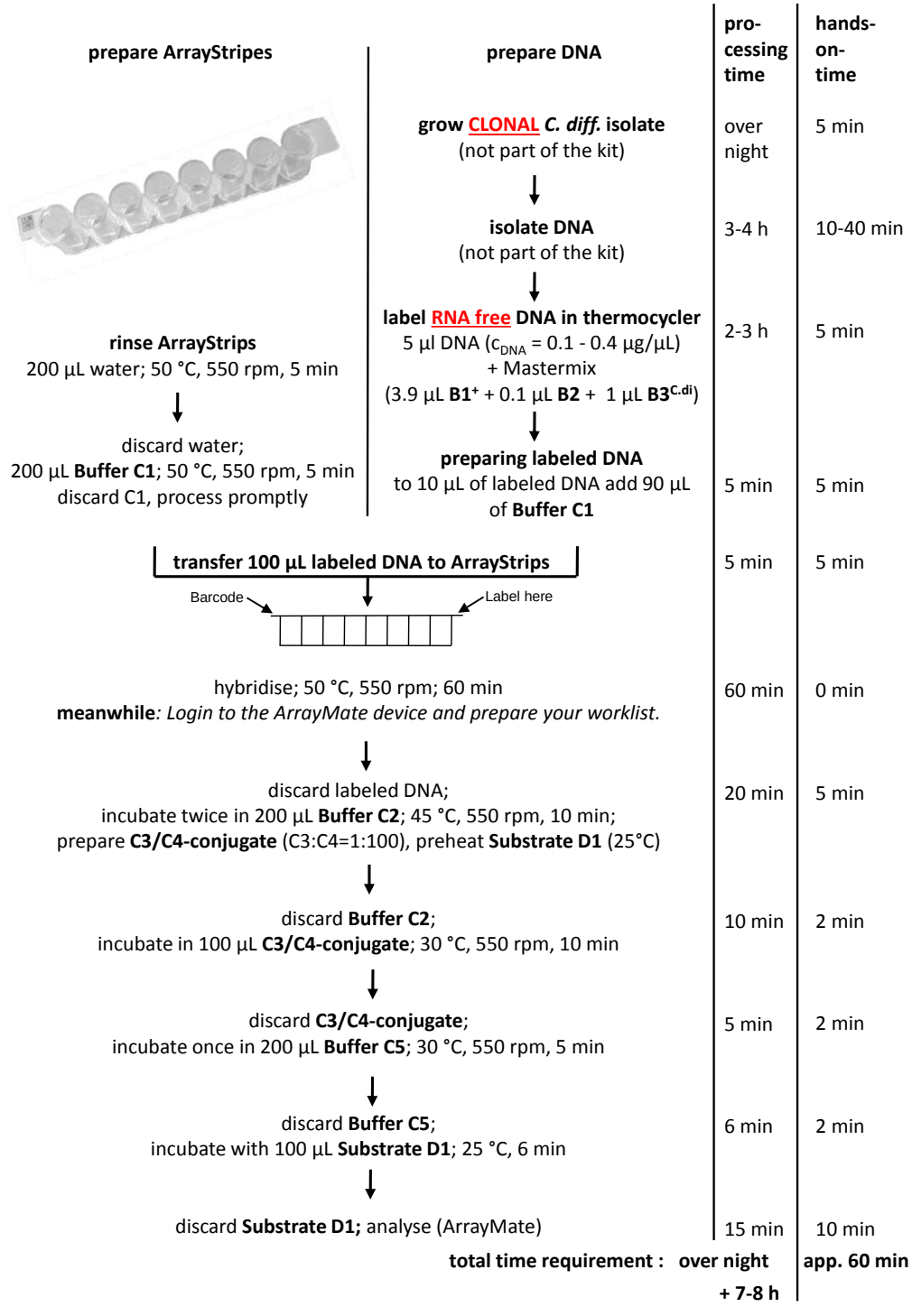
and/or http://alere-technologies.com/en/news.html

Currently available freeware programs are:

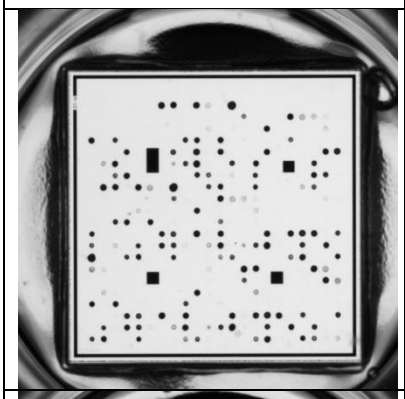
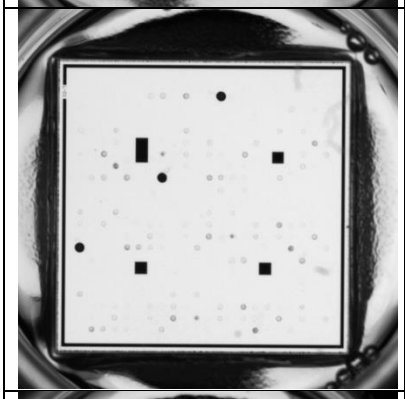
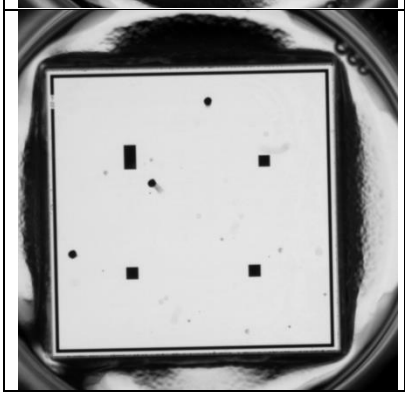
- Alere **Result Collector** for the conversion of multiple *result.xml files from the ArrayMate into spreadsheet tables. This should make it easier to compare isolates or to determine relative abundances of genes or strains etc.
- Alere **Worklist Generator** is a tool which helps you to create a well formatted worklist for the Arraymate.
- Alere **Report Generator** is a software tool to create reports using the assay software normally used and installed on the ArrayMate. It uses an image taken by the ArrayMate or a txt file (raw signal data file) and generates a report from the raw signal data.

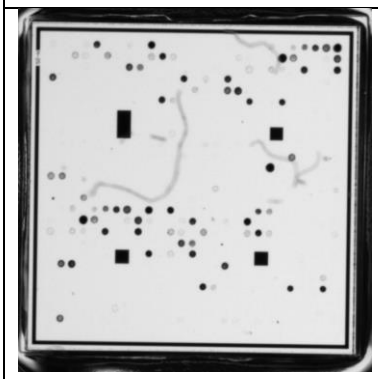
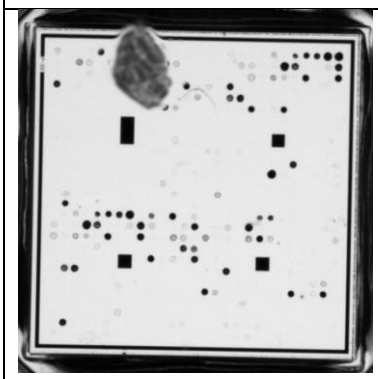
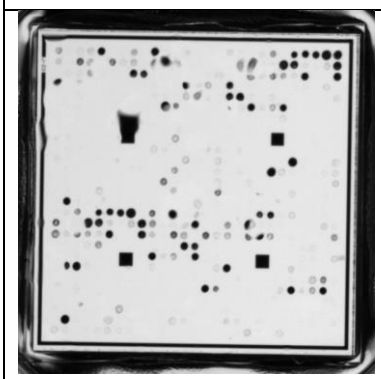
APPENDIX 1 – FLOW CHART

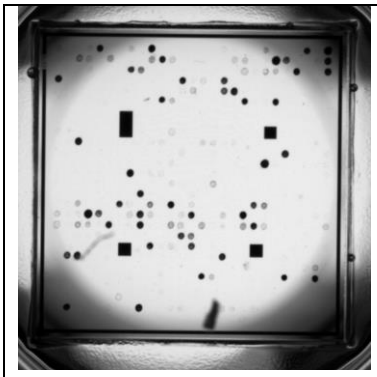
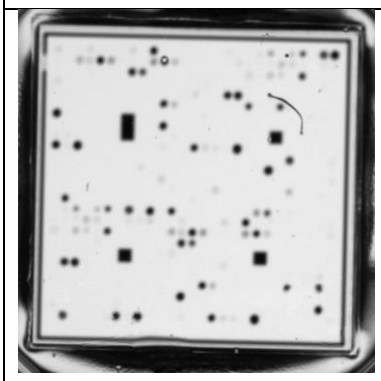
Quantifoil protocol. The figure on this page summarises the test procedure for the thermoshaker BioShake iQ by Quantifoil. Please always refer to the text section of this manual for further important details.


prepare ArrayStripes
prepare DNA
pro-cessing time
hands-on-time
grow CLONAL C. diff. isolate
(not part of the kit)
over night
5 min
isolate DNA
(not part of the kit)
3-4 h
10-40 min
label RNA free DNA in thermocycler
5 µl DNA (c_DNA = 0.1 - 0.4 µg/µL)
+ Mastermix
(3.9 µL B1+ + 0.1 µL B2 + 1 µL B3C.di)
2-3 h
5 min
rinse ArrayStrips
200 µL water; 50 °C, 550 rpm, 5 min
discard water;
200 µL Buffer C1; 50 °C, 550 rpm, 5 min
discard C1, process promptly
preparing labeled DNA
to 10 µL of labeled DNA add 90 µL
of Buffer C1
5 min
5 min
transfer 100 µL labeled DNA to ArrayStrips
Barcode
Label here
5 min
5 min
hybridise; 50 °C, 550 rpm; 60 min
meanwhile: Login to the ArrayMate device and prepare your worklist.
60 min
0 min
discard labeled DNA;
incubate twice in 200 µL Buffer C2; 45 °C, 550 rpm, 10 min;
prepare C3/C4-conjugate (C3:C4=1:100), preheat Substrate D1 (25°C)
20 min
5 min
discard Buffer C2;
incubate in 100 µL C3/C4-conjugate; 30 °C, 550 rpm, 10 min
10 min
2 min
discard C3/C4-conjugate;
incubate once in 200 µL Buffer C5; 30 °C, 550 rpm, 5 min
5 min
2 min
discard Buffer C5;
incubate with 100 µL Substrate D1; 25 °C, 6 min
6 min
2 min
discard Substrate D1; analyse (ArrayMate)
15 min
10 min
total time requirement :
over night
+ 7-8 h
app. 60 min

APPENDIX 2 – IMAGES FOR TROUBLESHOOTING

Image	Comment	Result sheets:
	A technically faultless, valid experiment.	Valid results, no error messages.
	This image is poor. This could be due to low DNA concentration, fragmented DNA, ethanol trace contaminations in DNA sample or expired reagents. The experiment should be repeated with a new DNA preparation. If this also fails, try an experiment with control DNA (CM).	The system will try to assign the isolate. It might yield a putative identification, than the "closest matching" Hybridisation Profile and the clade affiliation will be displayed. If this is not possible, and/or if species markers are missed, an error message will be displayed: "The signal intensity is low so that the image cannot reliably be analysed. This could be due to low DNA concentration, to fragmented DNA or presence of inhibiting substances. It might also be that the DNA was obtained from an isolate of another species than *C. difficile*."
	Species other than *C. difficile* tested or severe user error (no DNA, confusion of buffers, wrong temperatures…).	Check protocol. Check species identification and, if it was C. difficile, repeat with a new DNA preparation.

Contaminated/mixed culture:		If mutually exclusive alleles of species markers are detected simultaneoulsly, *i.e.*, if clones from different Clades are present, an error message will be displayed: "*The experiment is considered invalid due to over-staining, contamination and/or the presence of more than one clone of C. difficile. An assignment to a strain is not possible*." If two related clones (from one Clade) are present, this will most likely not be the case. The system might then provide a putative identification displaying the "closest matching" Hybridisation Profile and the Clade affiliation.
Image	**Comment**	**Handling**
	The bottom of the well is contaminated with dust particles.	Please clean the bottom of the well, scan and process again.
	The microarray surface is contaminated with dust particles.	If the microarray surface is contaminated with particles, wash the microarray with double distilled water (pipetting water carefully up and down, remove), scan and process again.
	The bottom of the well is contaminated with a liquid (e.g. buffer).	Please clean the bottom surface with a cleanroom wipe, scan and process again.

	Substrate D1 residue in the array edges.	Remove substrate D1 completely with a Pasteur pipette.
	Chip was not in focus during image acquisition.	Repeat image acquisition after fitting the ArrayStrip in the frame.

APPENDIX 3 – PROBE TO TARGET TABLE

CATEGORY	TARGET	PROBE ID
TOXINS	Toxin A	hp1132|tcdAp1
		hp1134|tcdAp2
		hp1135|tcdAp3
		hp1247|tcdAp2
	Toxin B	hp1118|tcdBp1
		hp1119|tcdBp1
		hp1121|tcdBp4
		hp1122|tcdBp4
		hp1124|tcdBp2
		hp1126|tcdBp2
		hp1127|tcdBp2
		hp1129|tcdBp3
		hp1130|tcdBp3
ANTIBIOTIC RESISTANCE	chloramphenicol acetyl transferase (cat)	hp1014|catD
	rRNA methyl- transferase (ermB)	hp1022|ermB
	tetracycline resistance protein,	hp1105|tetM
		hp1108|tetM
		hp1109|tetM
		hp1110|tetM
		hp1112|tetM
	streptogramin A acetyltransferase	hp1082|sat
		hp1083|sat
	lincomycin resist. protein lmrBNAP07	hp1096|lmrB
	lincomycin resist. protein lmrB630	hp1097|lmrB
LANTIBIOTIC RESISTANCE	lantibiotic two-component sensor histidine kinase	hp1079|spaK
		hp1080|spaK
	putative lantibiotic ABC transporter, permease protein	hp1251|spaE
		hp1252|spaE
EFFLUX SYSTEMS	bacitracin resistance protein locus 1	hp1013|bacA1
	bacitracin ATP binding cassette transporter	hp1071|bcrA
		hp1072|bcrA
		hp1073|bcrA
	ABC-type transporter, ATP-binding protein	hp1084|CD0456
	putative efflux pump	hp1087|cme
	MATE efflux family protein	hp1063|matE
	efflux pump antibiotic resistance protein	hp1098|ydiC
		hp1244|ydiC
vexP1/vncS	ABC-type transport system, permease Tn1549	hp1055|vexP1
		hp1059|vexP1
	two-component sensor histidine kinase Tn154c	hp1051|vncS
		hp1052|vncS
OTHER VIRULENCE GENES	C. difficile binary toxin component A	hp1023|cdtA
		hp1026|cdtA
		hp1027|cdtA
		hp1029|cdtA
	C. difficile binary toxin component B	hp1030|cdtB
		hp1031|cdtB
		hp1038|cdtB
		hp1039|cdtB
		hp1040|cdtB
		hp1041|cdtB
	channel-forming hemolysin	hp1103|hly3
	holin-like pore-forming protein	hp1113|tcdE

CATEGORY	TARGET	PROBE ID
	putative negative regulator of pathogenicity locus	hp1117|tcdC
CELL WALL PROTEINS	cellwall protein 66	hp1012|cwp66
		hp1016|cwp66
		hp1020|cwp66
		hp1021|cwp66
	cellwall protein 84	hp1018|cwp84
	splA Surface layer protein A	hp1150|slpA
		hp1151|slpA
		hp1153|slpA
		hp1154|slpA
		hp1155|slpA
		hp1156|slpA
		hp1158|slpA
		hp1164|slpA
		hp1166|slpA
		hp1167|slpA
		hp1168|slpA
		hp1169|slpA
		hp1170|slpA
		hp1171|slpA
		hp1173|slpA
		hp1174|slpA
		hp1175|slpA
		hp1176|slpA
		hp1177|slpA
		hp1178|slpA
		hp1180|slpA
		hp1182|slpA
		hp1183|slpA
		hp1184|slpA
		hp1186|slpA
		hp1188|slpA
		hp1190|slpA
		hp1191|slpA
		hp1192|slpA
		hp1193|slpA
		hp1195|slpA
		hp1197|slpA
		hp1198|slpA
		hp1199|slpA
		hp1200|slpA
		hp1201|slpA
		hp1202|slpA
		hp1203|slpA
		hp1204|slpA
		hp1205|slpA
		hp1206|slpA
		hp1208|slpA
		hp1209|slpA
		hp1210|slpA
		hp1211|slpA
		hp1232|slpA
		hp1233|slpA
		hp1234|slpA
		hp1236|slpA
		hp1237|slpA
		hp1239|slpA

CATEGORY	TARGET	PROBE ID
		hp1242|slpA
		hp1243|slpA
		hp1249|slpA
CELL DIVISON PROT.	septum formation initiation protein	hp1044|divIC
		hp1045|divIC
		hp1046|divIC
FLAGELLINS	flagellin subunit C	hp1141|fliC
		hp1142|fliC
		hp1144|fliC
		hp1145|fliC
		hp1147|fliC
MISCELLANEOUS GENES	putative endonuclease relaxase Tn1549-like	hp1214|CD0425
		hp1220|CD0425
		hp1228|CD0425
		hp1229|CD0425
		hp1230|CD0425
		hp1231|CD0425
	putative calcium-binding adhesion protein	hp1149|CD2797
		hp1212|CD2797
	ethanolamine iron-dependent alcohol dehydrogenase	hp1061|eutG
		hp1062|eutG
	leucine Rich repeat-containing domain protein	hp1227|IPR007253

APPENDIX 4 – TYPING INFORMATION

Definitions and Explanations

The displayed result will yield following typing information:

- Strains are named "HP-" (for Hybridization Profile) followed by a chronologically assigned number (see Table below). Isolates cluster into HPs or strains based on overall hybridization patterns with emphasis to *tcdA/B* and *slpA* alleles. Isolates or strains were regarded as one HP in case of at least 88% identity of positive/ambiguous/negative classifications for all probe positions covered, plus presence of identical *tcdA/B* and *slpA* alleles. Possibly mobile resistance markers are not considered for the definition of hybridization profiles or strains. It still needs to be clarified whether these genes could be used as subtyping markers for isolates within one HP (*i.e.,* for outbreak investigations). The list of recognized Hybridization Profiles can be expanded, and software updates will likely be provided on an annual basis. If you find new Hybridization Profiles, if you have array images of a strain not yet covered or if you have additional information on strain you wish to be included, please contact stefan.monecke@clondiag.com.
- Clade affiliation, as determined by overall profile. Clades were defined by Dingle, 2011 [2] in order as a taxonomic unit that includes related sequence types. Allelic variants of several markers depend on Clade affiliation, so that the Clade of an unknown isolate can be identified without actual sequencing.
- Sequence types associated with this strain/Hybridization Profile. This information is provided based on a database of isolates that have been typed in parallel by array and MLST. It is *not* directly derived from the actual experiment. If you have MLST results you want to be included, please contact stefan.monecke@clondiag.com.
- Ribotypes associated with this strain. This information is provided based on a database of isolates that have been typed in parallel by array and by ribotyping. It is *not* directly derived from the actual experiment. If you have results you want to be included, please contact stefan.monecke@clondiag.com.

- Assignment score. This is a score for the similarity of the actual experimental result to the predefined strain/Hybridisation Profiles. Scores below 87.25% exclude reliable strain identification, and could be attributed either to technical reasons or to the presence of a yet unknown strain but the the most similar strain and the Clade affiliation will be displayed. In case of a score below 83 %, the experiment is considered invalid, and both html files will display error messages. A value of 100% is unlikely because of the mobility of some genes in *C. difficile* and the possibility of "ambiguous" results for mismatching allelic probes.

List of Currently recognized Strains

Hybridi-sation profile	Fully sequenced strains (GenBank accession no.)	MLST Clade	Associated sequence types	*slpA* allele	Associated ribotypes	*tcdA/B* alleles	*cdtA/B* alleles	*cat*	*erm*(B)	*tet*(M)
HP-01	BI-9 (FN668944) QCD-63q42 (ABHD)	I	ST-03	$slpA_{BI9}$	RT-001, RT-015, RT-072	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	(var)	-
HP-02	ATCC43255 (ABKJ)	I	ST45, ST-46	$slpA_{BI9}$	RT-001, RT-013, RT-087	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	-	-
HP-03	-	I	ST-58	$slpA_{6407}$	RT-011, RT-049, RT-056	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	(var)	(var)
HP-04	-	I	ST-04	$slpA_{630}$	RT-137, RT-150	$tcdA_{R20291}$+ $tcdB_{630}$	($cdtA_{630}$+ $cdtB_{630}$)			
HP-05	-	I	N/A	$slpA_{DJNS0578}$	RT-163	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	-	-
HP-06	-	I	N/A	*slpA* negative	Unident. pattern	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	-	-
HP-07	-	I	N/A	$slpA_{R12884}$ trunc.	RT-054	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	-	-
HP-08	-	I	ST-55	$slpA_{R13711}$	RT-057, RT-070, RT-094	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-		
HP-09	-	I	ST-08	$slpA_{R13541}$	RT-002, RT-159	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	-	(var)
HP-10	-	I	N/A	$slpA_{23m63}$	N/A	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	-	-
HP-11	CD37 (AHJJ)	I	ST-03	$slpA_{23m63}$	RT-009	-	-			(var)
HP-12	-	I	N/A	$slpA_{JND08162}$	RT-103	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	-	-
HP-13	70-100-2010 (AGAC)	I	ST42	$slpA_{R12885}$	RT-014, RT-049	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	-	-
HP-14	-	I	N/A	$slpA_{Kohn}$	RT-015	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	-	-

Hybridisation profile	Fully sequenced strains (GenBank accession no.)	MLST Clade	Associated sequence types	*slpA* allele	Associated ribotypes	*tcdA/B* alleles	*cdtA/B* alleles	*cat*	*erm*(B)	*tet*(M)
HP-15	-	**I**	N/A	$slpA_{Kohn}$	N/A	-	-	-	-	-
HP-16	-	**I**	N/A	$slpA_{79685}$	RT-029	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	-	-
HP-17	-	**I**	N/A	$slpA_{JND09041}$	RT-064	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	-	(var)
HP-18	-	**I**	ST-17	$slpA_{MRY060211}$	RT-005	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	-	(var)
HP-19	-	**I**	N/A	$slpA_{J9952}$ trunc.	RT-013, RT-087	$tcdA_{R20291}$+ $tcdB_{630}$	-	-	-	-
HP-20	-	**I**	ST-08	$slpA_{R12884}$	RT-005, RT-045, RT-054	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	-	-
HP-21	-	**I**	N/A	$slpA_{R13711}$	RT-031	-	-	-		
HP-22	-	**I**	N/A	$slpA_{R13711}$	N/A	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	pos	pos
HP-23	-	**I**	ST-54	$slpA_{R13711}$	RT-012	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	-	pos	pos
HP-24	Strain 630 (AM180355) Strain 6534 (ADEJ)	**I**	ST-54	$slpA_{630}$	RT-012	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{630}$+ $cdtB_{630}$	(var)	(var)	(var)
HP-25	-	**I**	ST-35	$slpA_{JND08037}$	RT-046	$tcdA_{R20291}$+ $tcdB_{630}$	-	pos	pos	pos
HP-26	-	**I**	N/A	$slpA_{1446}$	RT-039	-	-	-	pos	pos
HP-27	-	**I**	N/A	$slpA_{R13700}$	RT-010	-	-	-	pos	-
HP-28	Strain 6407 (ADEH)	**I**	ST-58 or related	$slpA_{6407}$	N/A	$tcdA_{CF5}$+ $tcdB_{630}$	-	-	-	-
HP-29	-	**I**	N/A	$slpA_{6407}$	RT-071	-	-	-	-	-
HP-30	-	**I**	ST-09	*slpA* negative (probe-1164 sometimes ambiguous)	RT-029, RT-081, RT-094	$tcdA_{R20291}$+ $tcdB_{630}$	-	-	(var)	-
HP-31	CD196 (FN538970) BI1 (FN668941) CIP107932(ABKK) QCD-76w55(ABHE) QCD-97b34(ABHF)	**II**	ST-01	$slpA_{R20291}$	RT-027	$tcdA_{R20291}$+ $tcdB_{R20291}$	$cdtA_{R20291}$+ $cdtB_{R20291}$	-	-	-
HP-32	R20291 (FN545816) 2007855 (FN665654) QCD-32g58 (AAML) QCD-37x79 (ABHG) QCD-66c26 (ABFD)	**II**	ST-01	$slpA_{R20291}$	RT-027	$tcdA_{R20291}$+ $tcdB_{R20291}$	$cdtA_{R20291}$+ $cdtB_{R20291}$	-	(var)	-
HP-33	-	**III**	N/A	$slpA_{R12884}$	RT-023	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{Clade\ III}$+ $cdtB_{R20291}$	-	(var)	-
HP-34	-	**III**	N/A	$slpA_{R12884}$ trunc.	N/A	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{Clade\ III}$+ $cdtB_{R20291}$	-	-	-
HP-35	CF5 (FN665652) 002-P50-2011 (AGAA) 050-P50-2011 (AGAB) M68 (FN668375)	**IV**	ST-37, ST-86	$slpA_{CF5}$	RT-017	$tcdA_{CF5}$+ $tcdB_{CF5}$	-	-	(var)	(var)

Hybridi-sation profile	Fully sequenced strains (GenBank accession no.)	MLST Clade	Associated sequence types	*slpA* allele	Associated ribotypes	*tcdA/B* alleles	*cdtA/B* alleles	*cat*	*erm*(B)	*tet*(M)
HP-36	-	**IV**	N/A	$slpA_{79685}$	RT-017	$tcdA_{CF5}$+ $tcdB_{CF5}$	-	-	-	-
HP-37	M120 (FN665653) NAP07 (ADVM) NAP08 (ADNX)	**V**	ST11	$slpA_{R13540}$	RT-078	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{R20291}$+ $cdtB_{M120}$	-	(var)	(var)
HP-38	Strain 6466 (ADDE)	**V**	ST-11 or related	$slpA_{R13540}$	N/A	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{R20291}$+ $cdtB_{M120}$	-	-	pos
HP-39	QCD-23m63 (ABKL)	**V**	ST-11 or related	$slpA_{23m63}$	N/A	$tcdA_{R20291}$+ $tcdB_{630}$	$cdtA_{R20291}$+ $cdtB_{M120}$	-	-	-
HP-40	Strain 6503 (ADEI)	**"VI"**	ST127 dlv	$slpA_{6503}$	N/A	-	-	-	-	-